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VIRGINIA RECREATIONAL FISHING DEVELOPMENT FUND
SUMMARY PROJECT APPLICATION*

NAME AND ADDRESS OF APPLICANT:

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PRIORITY AREA OF CONCERN:

Mycobacteriosis in striped bass (*Morone saxatilis*) of Chesapeake Bay

PROJECT LOCATION:

Chesapeake Bay, USA (baywide survey)

DESCRIPTIVE TITLE OF PROJECT:

Monitoring Mycobacteriosis in Chesapeake Bay Striped Bass *Morone saxatilis*: Tracking the State of the Epizootic

PROJECT SUMMARY:

This is a continuing large temporal- and spatial-scale survey of mycobacterial disease in Chesapeake Bay striped bass using the VIMS ChesMMAP trawl survey as a sampling platform. Striped bass will be collected at 80 randomized stations throughout the Bay five times per year (2006-2007). The prevalence and severity of mycobacteriosis will be determined by histological techniques, and molecular diagnostic tools will be used to determine the species of mycobacteria present in diseased fish, as well as to detect cryptic infections in the absence of disease. Environmental data will be taken at each station, and standard data will be taken on each fish, including morphometrics, otolith-determined age, and gut contents. These data will be combined to provide a much better understanding of mycobacteriosis in striped bass at a population level. In addition to continuing survey efforts, we will also conduct a preliminary histological and molecular survey of mycobacteriosis in recreationally and commercially important finfish other than striped bass, and will conduct a pilot study to detect the presence of mycobacteria in the water column and sediment samples.

EXPECTED BENEFITS:

Striped bass are of huge economic and cultural importance to recreational and commercial anglers. The current epizootic of mycobacteriosis has generated a large amount of concern in those who have an interest in fishing and the health of the Bay in general. Therefore, up-to-date and accurate information about the current state of the epizootic is of importance to researchers, fisheries managers, and the general public. The ChesMMAP survey used in this study is currently the most comprehensive existing platform for monitoring the status of the mycobacteriosis epizootic in Chesapeake Bay. Continued funding of mycobacteriosis survey efforts using the ChesMMAP platform will allow continuation of a high-quality 4 year dataset that will provide information on the prevalence, severity, and etiologic agents of disease in striped bass over large temporal and spatial scales. In addition, preliminary studies on mycobacterial distribution in other finfish species and in environmental samples will provide valuable information about the ecology of *Mycobacterium* spp. in the Chesapeake Bay, and will augment our overall understanding of disease in striped bass.

COSTS:

The ChesMMAP program is currently fully funded through federal and state sources, so the major costs for the collection platform are already covered. Funding of this project will therefore provide the benefit of baywide sampling coverage for the relatively minor costs of sampling equipment, laboratory processing, and reagents. This project is being submitted for joint consideration by the RFAB and CFAB.

VMRC Funding:	$59,312
Recipient Funding:	$17,784
Total Costs:	**$77,096**

Detailed budget must be included with proposal.

Updated 6/1/05

***This form alone does not constitute a complete application, see application instructions or contact Sonya Davis at 757-247-8155 or sonya.davis@mrc.virginia.gov : Due dates are June 15 (Jul. – Nov. Cycle) and December 15 (Jan. – May Cycle)**

Proposed Budget: Monitoring Mycobacteriosis in Chesapeake Bay Striped Bass *Morone saxatilis*: Tracking the State of the Epizootic

Category	MRFAB	VIMS	Total
Personnel			
Gauthier: PI (15%/5%)	6,000	2,000	8,000
Vogelbein: Co-PI (5%/3%)	4,000	2,400	6,400
Reece: Co-PI (6%)	3,600		3,600
Lab. Specialist (30%)	7,800		7,800
Fringe Benefits	5,350	1,320	6,670
Supplies			
Reagents, sampling supplies	8,200		8,200
Histology	6,500		6,500
Real-time PCR reagents	5,000		5,000
Travel			
Field sites, meetings $0.55/mi (VIMS) $0.325/mi (personal vehicle)	1,000		1,000
Facilities & Administrative Costs	11,862	12,064	23,926
TOTAL	**$59,312**	**$17,784**	**$77,096**

Facilities and Administrative Costs:
F&A Costs capped at 25% for funds requested from VMRC/RFAB program.

Monitoring Mycobacteriosis in Chesapeake Bay Striped Bass *Morone saxatilis*: Tracking the State of the Epizootic

A Proposal Submitted to the
Virginia Recreational Fishing Advisory Board
and
Virginia Commercial Fishing Advisory Board

By

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Sponsored Research

Introduction

The anadromous striped bass, *Morone saxatilis*, is one of three dominant piscivores in the Chesapeake Bay and fills a critical ecological niche in estuarine food webs (Hartman & Brandt 1995). Disease outbreaks have been reported in Chesapeake Bay striped bass since the late 1980s. Mortality in fish from Maryland waters of the bay in 1988 was associated with *Streptococcus* spp. (Baya et al. 1990), and visceral and dermal lesions observed in 1994 were attributed to *Edwardsiella* infections (Baya et al. 1997). Beginning in 1997, striped bass exhibiting poor body condition and ulcerative skin lesions were observed in Virginia and Maryland waters of Chesapeake Bay**.** Histopathology revealed granulomatous inflammation associated with acid-fast bacteria, consistent with infection by *Mycobacterium* spp. (Vogelbein et al. 1999). Subsequent surveys of mycobacteriosis in Chesapeake Bay striped bass have demonstrated high visceral and dermal disease prevalence in Bay waters (Vogelbein et al. 1999, Cardinal 2001, Overton et al. 2003, Gauthier 2006, in prep). Mycobacteriosis has previously been described in wild and cultured striped bass from the US Pacific Coast, with prevalences reaching 68% and 80%, respectively (Sakanari et al. 1983, Hedrick et al. 1987). Hybrid striped bass (*Morone chrysops* × *Morone saxatilis*) are also known to be susceptible from spontaneous infections observed in aquaculture and via experimental exposures (Wolf & Smith 1999, Bowser et al. 2004).

Mycobacteriosis is a subacute to chronic disease common in wild and captive fishes worldwide. Mortality is not typically associated with mycobacteriosis in wild finfish populations, however, this may be attributed to the difficulties in observing protracted mortalities from a chronic disease in a field setting. High mortality is commonly observed in aquaculture (Nigrelli & Vogel 1963, Hedrick et al. 1987, Bruno et al. 1998). *Mycobacterium marinum, M. fortuitum* and *M. chelonae* are the most frequently cultured isolates from diseased fishes, although several other species have been reported (Lansdell et al. 1993, Tortoli et al. 1996, Herbst et al. 2001, Rhodes et al. 2004). In most cases, disease is visceral, with spleen, liver and kidney being the primary target organs. Granulomatous inflammation, often with extensive tissue destruction, is characteristic, although more poorly organized inflammatory responses are observed, typically in association with high bacillary loads (Kent et al. 2004). External clinical signs include scale loss, skin ulceration, emaciation, exophthalmia, pigmentation changes and spinal defects (Nigrelli & Vogel 1963, Snieszko 1978, Wolke & Stroud 1978, Bruno et al. 1998).

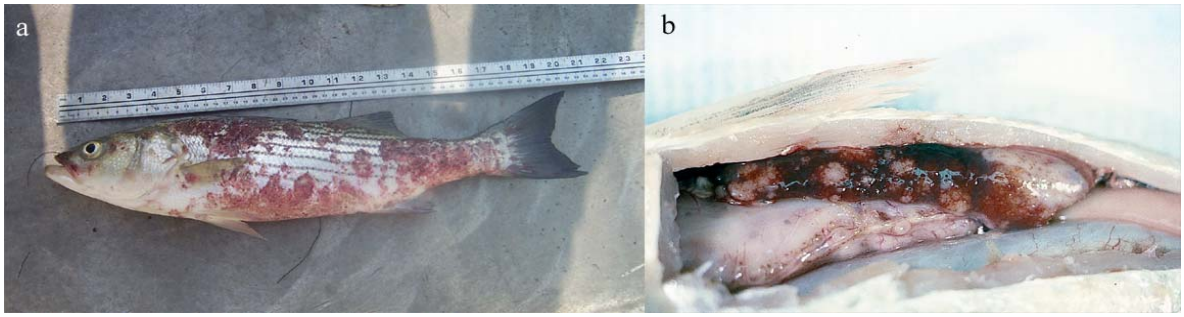


Fig. 1: Gross clinical signs of mycobacteriosis in Chesapeake Bay striped bass. a) severe ulcerative dermatitis. Note shallow, hemorrhagic and hyper-pigmented (dorsal) ulcers. b) Multi-focal gray nodules (arrows) within the spleen.

Characterization and identification of etiologic agent(s) associated with the epizootic of mycobacteriosis in Chesapeake Bay striped bass are ongoing. A number of isolates have been cultured, including the new species, *M. shottsii* and *M. pseudoshottsii,* the pathogen *M. marinum,* and species typically considered to be saprophytes (e.g., *M. gordonae*) (Rhodes et al. 2001, Rhodes et al. 2003, Rhodes et al. 2004, Rhodes et al. 2005). Other isolates resemble but do not exactly match known phenotypic profiles, and potentially represent new species (Rhodes et al. 2004) . *Mycobacterium shottsii* and *M. pseudoshottsii* are the most common isolates recovered from diseased striped bass, and co-infections with multiple mycobacterial species occur. Both *M. shottsii* and *M. pseudoshottsii* are closely related to *M. marinum* and *M. ulcerans*, important pathogens of fishes and humans, respectively.

Recently, researchers have reported a decline in striped bass tagging returns, suggesting that natural mortality of striped bass stocks in Chesapeake Bay has increased in recent years (Kahn 2004). The causes for this apparent increase in natural mortality are unknown, but the high observed prevalence of mycobacteriosis in Chesapeake Bay striped bass, as well as the decreased condition factor observed in many heavily infected fish, suggests that this

disease may be at least partly responsible. Our research group at VIMS has an ongoing Sea Grant and RFAB-funded tagging program designed to determine whether or not diseased fish are experiencing increased mortality in Chesapeake Bay. In addition to the possibility that mycobacteriosis may be causing increased mortality among striped bass in Chesapeake Bay, skin lesions of mycobacterial origin have been reported with increasing frequency in these fish since 1997. Lesions can be highly disfiguring, to the point that anglers frequently do not wish to handle affected fish. Although negative impacts of skin disease on the recreational fishery cannot easily be measured, the product quality of this popular fishery is clearly being affected.

Prior surveys of mycobacteriosis in Chesapeake Bay striped bass have been greatly limited in terms of spatial and temporal coverage. Additionally, surveys relying on pound nets or hook-and-line sampling methodologies are subject to serious collection biases which may lead to erroneous conclusions. Therefore, extension of the findings of previous surveys to interpretation of the epizootiology of mycobacteriosis on a Bay-wide scale is problematic. Since March 2003, we have been collaborating with the Chesapeake Bay Multispecies Monitoring and Assessment Program (ChesMMAP) through the Fisheries Department at VIMS (http://www.fisheries.vims.edu/chesmmap/). This federal- and state-funded program is a large-scale trawl survey which samples adult fish five times per year over the entire Bay, ranging from the Bay mouth to Poole's Island. Sampling is performed in twenty-minute tows at 80 stations per cruise, with a stratified-random design that covers all depths of the Bay. Data collection on fish includes basic morphometrics (length, weight), otolith-determined age, and gut content analysis. In addition, comprehensive environmental data are collected at each station. The ChesMMAP survey is intended to develop data on the distribution and feeding habits of finfish species in Chesapeake Bay, and to apply these data to development of comprehensive multispecies management models. By virtue of its design and scale, this survey also provides an ideal sampling platform for studying disease in wild fishes. Consequently, we have been using the ChesMMAP platform to survey the presence and severity of splenic mycobacteriosis, as well as presence of skin lesions. This project, which was supported by the VMRC for 2005-2006, has allowed monitoring of disease in a large number of striped bass representing both sexes in most age-classes, with excellent spatial and temporal coverage over the entire Chesapeake Bay. Prevalence and severity data from 2003-2005 are currently being compiled in a manuscript that will provide managers and the public with the most comprehensive analysis to date of mycobacteriosis in striped bass.

One of the major difficulties in understanding mycobacteriosis in Chesapeake Bay striped bass is that it does not appear to be a simple case of one bacterial species causing the disease. Multiple *Mycobacterium* spp. have been cultured from both diseased and healthy Chesapeake Bay striped bass, either individually or in combination (Rhodes et al. 2004). This strongly suggests that more than one species of mycobacteria is involved in production of disease. The limited number of fish from which cultures have been obtained, however, has not led to definitive understanding of which species cause the disease, and which species are responsible for disease on a Bay-wide scale. Detection and species identification of mycobacteria in a large number of fish over the entire area of the Bay is necessary in order to shed light on these questions. Because of the prohibitive time requirements of standard culture and the logistical difficulties of aseptic sampling on a large scale, accomplishment of these goals requires use of rapid molecular diagnostic tools. With previous support from the VMRC/RFAB, we have developed Polymerase Chain Reaction/Restriction Fragment Length Polymorphism (PCR/RFLP) assays capable of detecting and differentiating species of *Mycobacterium* from striped bass tissues. These techniques have been used to analyze a large number of striped bass collected in 2005, and will shortly be applied to samples collected in 2006. A final report on these molecular probes will be delivered to the RFAB in November 2006, and we anticipate a publication detailing their development and optimization will be submitted to a peer-reviewed journal within the year.

We propose here to continue large-scale monitoring of mycobacteriosis in Chesapeake Bay striped bass using the ChesMMAP platform. As in previous years, presence and severity of mycobacterial disease will be determine via histological methods, and molecular tools (PCR/RFLP) will be applied to detect cryptic infections, and to determine the *Mycobacterium* spp. present in striped bass. The large numbers of samples examined with PCR/RFLP in 2005-2006 will provide sufficient information to determine what species of *Mycobacterium* are primarily associated with disease in striped bass. Therefore, sampling for molecular analyses will be scaled back and used in a monitoring capacity to determine if shifts in the *Mycobacterium* spp. infecting striped bass occur from year to year. This will provide further information as to the species that will be of primary interest for further field and laboratory studies.

In addition to continuing survey, we propose to extend our sampling effort to examine finfish species other than striped bass for the presence of mycobacterial infection and disease. This will allow us to identify other

recreationally and commercially important finfish species at risk for disease, as well as to identify prey species that may be transmitting mycobacteria to striped bass. Finally, using a molecular technique called real-time PCR, we will conduct a pilot study to determine whether detectable levels of *M. pseudoshottsii* are present in the water column in the mainstem Bay and sediments in the York and Rappahannock rivers. This pilot study represents a crucial first step toward answering important questions about the ecology of *Mycobacterium* spp. in the Chesapeake Bay

Current Research Needs

Recently, a NOAA- and USGS-sponsored workshop entitled "Mycobacteriosis in Striped Bass" was convened in Annapolis, MD (9 May-11 May, 2006). The workshop included research scientists from various federal and state agencies and fisheries managers involved with mycobacteriosis-related research and potential management. Among the numerous research priorities identified by this workshop, continued comprehensive monitoring of the disease epizootic was preeminent. This activity is considered crucial to continuing research on mycobacteriosis, as it provides scientists and managers with current information on this potential impact to the striped bass fishery, identifies potentially fruitful areas for further research, and establishes a baseline by which the success of future management strategies may be measured. The ChesMMAP-based mycobacteriosis survey that we describe here is by far the most comprehensive program currently in place for these purposes. Consequently, we feel that this program will be pivotal in future research and management efforts, and that maintaining a continuous data set is of significant present and future value. In addition to continued monitoring, a better understanding of the ecology of *Mycobacterium* spp. in Chesapeake Bay was identified as a main research priority. This includes determination of whether the same *Mycobacterium* spp. infecting striped bass are also present in other finfish, as well as answering the question of whether mycobacteria such as *M. pseudoshottsii* are present in the environment, or if they are obligate pathogens, only found within fish hosts.

From prior studies, it is known that mycobacteriosis affects a large portion of the striped bass population of Chesapeake Bay. In order to provide meaningful information about a chronic disease process such as mycobacteriosis, it is crucial to collect comprehensive data on a multi-year timeframe. Prior to the initiation of these studies, surveys for mycobacteriosis suffered from limitations in spatial and temporal coverage, significant sampling bias, and spotty year-to-year coverage. While these studies yielded useful information, they are of limited value for understanding the disease situation in Chesapeake Bay on a population level. The continuing studies proposed here are extremely valuable in that they are spatially and temporally comprehensive, and build on an existing 4 year (2003-2006) dataset. The proposed studies are also comprehensive in their cross-sectional coverage of the striped bass population, including adequate coverage of both sexes and all age groups. As will be demonstrated in the final section of this proposal, such coverage is necessary for understanding disease epizootiology in the striped bass population as a whole.

Analysis of data collected from 2003-2005 indicates that, when several variables are taken into account, the disease situation in the Bay was constant during this time period (logistic regression, $p>0.05$ of covariate "year"). This is a significant finding, as it differs from largely anecdotal reports that disease is increasing in the Bay. This underscores the value of a comprehensive sampling platform for providing accurate information regarding the disease epizootic. The observation of consistent disease expression over several years may on one hand be interpreted as evidence that further disease survey efforts are unnecessary. Because mycobacteriosis is a chronic disease, however, disease may cycle over longer time frames which require multiple years of data to become recognizable. Additionally, if prevalence of mycobacteriosis is linked to environmental variables such as geographical extent of seasonal hypoxia, multiple years of data will be necessary to discern these trends. Perhaps more importantly, continuing monitoring is necessary so that major shifts in the prevalence or severity of disease will be observed if they occur in the future. Such shifts in prevalence potentially have implications for the overall health of the striped bass stock, especially if mycobacteriosis plays a significant role in natural mortality. VMRC/RFAB- and Sea Grant-funded studies examining the question of whether mycobacteriosis causes significant mortality in Chesapeake Bay striped bass are currently ongoing, and will be directly applicable to interpretation of data gained by this proposed study.

Since 1997, the association between emaciation ("skinny stripers") and severe mycobacterial disease has been noted by researchers and anglers alike. Dissecting this association is somewhat of a "chicken-or-the-egg" problem. On one hand, disease may be causing striped bass to become emaciated, while on the other, lack of forage fish such as

menhaden may be leading to decreased condition in striped bass and increased susceptibility to disease. Certain parts of this complicated question can only be answered by laboratory studies. Field studies, however, are still necessary to directly observe the relationships between diet, condition, and disease in wild striped bass. The ChesMMAP survey collects information on gut contents of all fish collected, including striped bass. We are currently conducting preliminary analyses of the association between diet and disease status, and are observing some interesting trends that indicate the diet of diseased striped bass may differ from that of healthy fish. The diet of diseased fish appears to be shifted to lower-value prey items such as worms and anchovies, whereas more high-value prey items (e.g. menhaden) appear to be consumed by healthy bass. If this trend were to be seen consistently from year-to-year, it would clarify the association between condition and disease, in that it would indicate a change in behavior of diseased fish.

The ability of *Mycobacterium* spp. to infect and cause disease in a wide variety of finfish species is well documented (Nigrelli & Vogel 1963). The presence of mycobacteriosis in Chesapeake Bay fishes other than striped bass, however, remains largely unknown. Additionally, the presence of *M. shottsii* and *M. pseudoshottsii* in species other than striped bass has never been assessed. As we continue our efforts to understand mycobacteriosis in striped bass, it is crucial that we gain information as to the presence of potentially infectious mycobacteria in prey species. It is also important to assess the impact of mycobacteriosis on other recreationally and commercially important finfishes in order to determine if species other than striped bass are potentially at risk. The proposed study will examine a number of finfish species, including weakfish, summer flounder, croaker, spot, white perch, and menhaden for the presence of mycobacterial infection and disease. Further, it will be determined whether these species are infected with the same species of mycobacteria affecting striped bass.

In addition to detection of *Mycobacterium* spp. in finfish species other than striped bass, it is crucial that we begin to examine the distribution of mycobacterial species of interest in the environment. To date, all mycobacteria associated with disease in fish (e.g. *M. marinum*) are "environmental mycobacteria," so-called because of their ability to persist and replicate outside of an animal host. We may assume a similar ecology for species infecting striped bass, especially *M. shottsii* and *M. pseudoshottsii*. Some mycobacteria of importance in human disease, however, including *M. tuberculosis*, *M. bovis*, and *M. leprae*, are obligate pathogens, which are not found in the environment and only exist within animal hosts. Despite the obvious importance of knowing which of these lifestyles is the case for *M. shottsii* and *M. pseudoshottsii*, no attempts have been made to detect these species outside animal hosts. If it is determined that these species are found in the environment, it will be possible to look for areas in which they are most highly concentrated, hydrogeographical features that may influence their distribution, and anthropogenic influences (e.g. land use) that may influence their density. If these species are not found in the environment, suggesting they are obligate pathogens, this information would be critical in future efforts to model the epizootiology of mycobacteriosis in striped bass populations. We propose here to conduct a small pilot study to determine whether *M. pseudoshottsii* can be detected in either water samples collected during the course of the ChesMMAP survey, or sediment samples collected from the York and Rappahannock rivers.

Objectives

1. Collect data on the prevalence and severity of mycobacterial disease in Chesapeake Bay striped bass with broad spatial (Bay-wide) and temporal (March-November) coverage. Use these data to augment 4 years of existing data and examine disease trends in the striped bass stock as a whole.

2. Examine the relationships between mycobacteriosis and fish host characteristics (e.g. fish age, sex, year-class) as well as time of year and geographical location, in order to determine risk factors for development of disease.

3. Apply recently developed, RFAB funded molecular tools to detect and determine species of mycobacteria in Chesapeake Bay striped bass. Use these data to determine spatial and temporal distribution of individual *Mycobacterium* spp. infecting striped bass, as well as to indicate the species of mycobacteria most commonly associated with diseased fish.

4. Examine finfish species other than striped bass for mycobacterial infection and disease. Use molecular techniques to determine the *Mycobacterium* spp. present in these fishes, and determine whether they are the same organisms associated with disease in striped bass.

5. Conduct a small pilot study using real-time PCR methods to detect *M. pseudoshottsii* in water and sediment samples.

6. Continue to provide managers with much-needed information on the basic pathobiology of and risk factors associated with mycobacteriosis in striped bass so that potential strategies to ameliorate the disease in Chesapeake Bay can be formulated.

Approach

Striped bass will be collected via otter trawl aboard the R/V Bay Eagle during the 2006-2007 ChesMMAP survey. Comprehensive environmental data will be gathered for each station. The ChesMMAP survey covers 80 stations 5 times per year at randomized locations ranging from the Bay mouth to Poole's Island. Approximately 500 striped bass per year were sampled by the ChesMMAP survey from 2003-2005, and we anticipate similar numbers will be sampled during 2006-2007. Approximately 200 striped bass will also have tissues taken for PCR/RFLP analysis. Additionally, we will sample 25 fish each from the following species list: summer flounder (*Paralichthys dentatus*), weakfish (*Cynoscion regalis*), spot (*Leiostomus xanthurus*), croaker (*Micropogonias undulatus*), white perch (*Morone americana*), and menhaden (*Brevoortia tyrannus*). This sample size is sufficient to detect 10% prevalence with 90% certainty, which we believe to represent an adequate tradeoff between sample precision and species coverage. Summer flounder and weakfish are included in this list due to their economic and ecological importance, as well as their status as prey species to larger striped bass. Spot, croaker, and menhaden are included primarily due to their importance as prey species. White perch are included due to their abundance and close taxonomic relationship to striped bass. If time and supplies permit, other potential prey species such as butterfish, alewife, and blueback herring will be included in the sampling survey.

Upon capture in the trawl and collection of morphometric data and otoliths, the peritoneal cavity of fishes will be accessed via ventral incision. Using sterile disposable sampling apparatus (forcep/scalpel), a small sample of the spleen will be taken and preserved in 95% ethanol for PCR analysis. The remainder of the spleen will be excised and fixed in 10% Z-Fix buffered formalin for histology. After fixation, the spleen will be divided and placed in a histological cassette so that six transverse sections of each spleen will be analyzed. This approach offers significantly better sampling coverage than the single section that is typically examined for this type of analysis. After routine histological processing, spleen sections will be examined for the presence and number of lesions. Images of individual spleens will be taken using a microscopic image collection system, and splenic area will be determined using image analysis software (MetaMorph). Disease severity index (SI) will be calculated as lesions/mm^2.

For PCR/RFLP analysis, DNA will be extracted from ethanol-fixed tissue with a commercially available kit (DNeasy, Qiagen). Mycobacterial large subunit ribosomal (LSU) DNA will be first be amplified with genus-specific primers. Positive samples will then be re-screened with sub-genus-specific primers which identify mycobacteria as either Group 1 (including *M. shottsii*, *M. pseudoshottsii*, and *M. marinum*) or Group 2 (*M. triplex*-like). RFLP will then be performed on positive PCR amplicons to determine species. A schematic of the PCR/RFLP assay is given in Fig. 2. The specificity of Group 1 primers, and RFLP "fingerprints" of Group 1 *Mycobacterium* spp. are shown in Fig. 3.

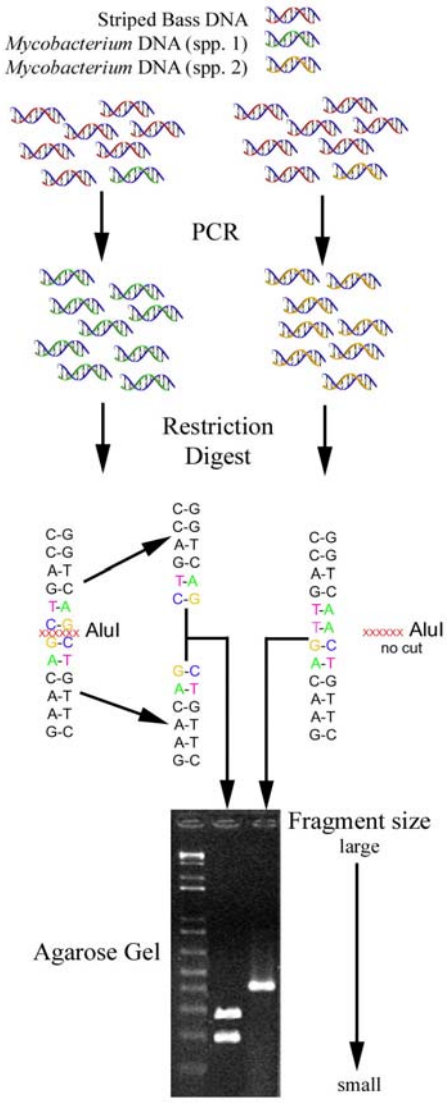
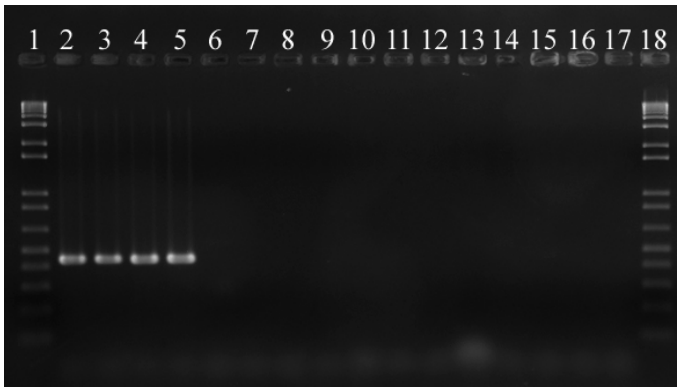


Fig. 2: Diagram of PCR/RFLP technique. Mycobacterial DNA is first specifically amplified from mixture of extracted striped bass and mycobacterial DNA (spleen sample). Amplified mycobacterial DNA is then subjected to digestion with specific restriction enzymes (AluI in this example). Restriction enzymes cut DNA at very specific sequences, in this example at TCGA. Because species 1 has this sequence and species 2 does not (species 2 sequence is T*T*GA), AluI cuts DNA from species 1 into two fragments, while DNA from species 2 remains intact. When the digested DNA is run on an agarose gel, which separates DNA fragments by size, one large fragment is seen for species 2, while two smaller fragments are seen for species 1.

In addition to PCR/RFLP species determination as described above, we have developed methodologies to deal with co-infections, where an individual fish is infected with more than one species of mycobacteria. Due to a phenomenon called "primer competition," in situations of a co-infection, only the most abundant species of mycobacteria is detected via PCR/RFLP. We have developed additional primer sets, however, that preferentially amplify certain species of mycobacteria, as well as primer sets that only amplify *M. pseudoshottsii*, based on the presence of a unique gene target, IS2404. By running additional PCR reactions on positive samples, we are therefore able to resolve co-infections with *M. shottsii* and *M. pseudoshottsii*, the most common mycobacteria detected in Chesapeake Bay striped bass. Frequency of detection of various *Mycobacterium* spp. from spleen samples collected during a joint USGS/ChesMMAP effort during spring and fall 2005 is given in Table 1.

Real-time PCR analysis will be run to detect *M. pseudoshottsii* in water and sediment samples. *M. pseudoshottsii* is selected here as a target species due to its high prevalence (see Table 1) in 2005-2006 samples, and the presence of a unique gene target, IS2404, in its genome (Rhodes et al. 2005). Real-time PCR is similar to regular PCR, which is the technique used in the PCR/RFLP analyses described above, but differs in that a fluorescent dye is included in the reaction mixture. This dye combines with double-stranded DNA, which increases in concentration as the PCR reaction continues. The reaction is carried out in a specialized thermal cycler that monitors the increase in fluorescence in real-time. This allows not only detection of the target gene, but by monitoring how quickly fluorescence crosses the background threshold, the original gene target may be quantified by comparison to a standard curve (see Figure 4). Therefore, by using real-time PCR, we will be able to detect and quantify *M. pseudoshottsii* in water and sediment samples if it is present. Real-time PCR also has an advantage in sensitivity over standard PCR, which will be useful in detecting very low levels of *M. pseudoshottsii* in environmental samples.

a)



b)

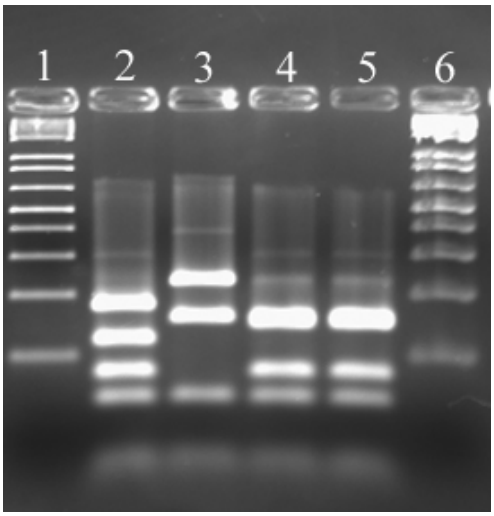
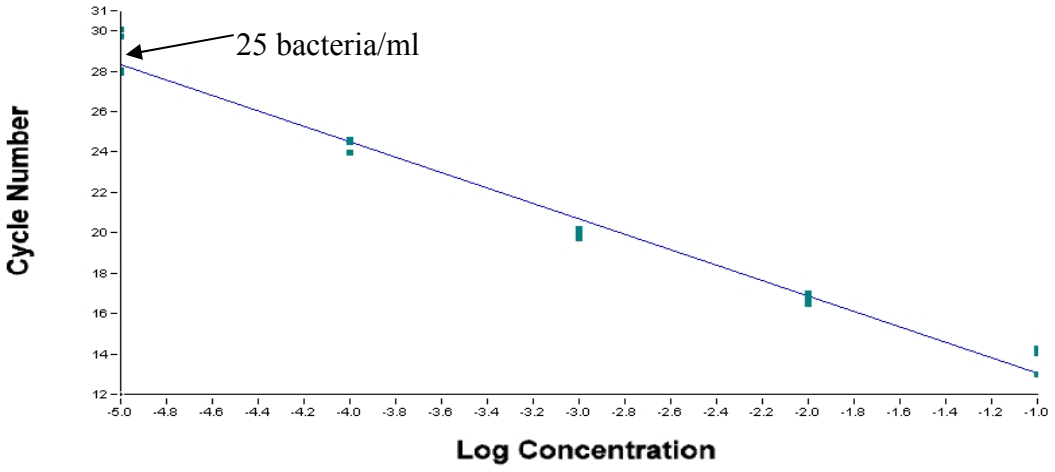


Fig. 3a. Specificity of Group 1 primers. Gel shows nested secondary amplification product after primary amplification with genus-specific primers. Lanes 1 and 18 are size standards. Lanes 2-5 are *M. shottsii*, *M. pseudoshottsii*, *M. marinum* (VIMS isolate R106), and *M. marinum* (ATCC#BAA535). Subsequent lanes are (6) *M. triplex* (ATCC #700071), (7-12) *M. triplex*-like mycobacteria (VIMS #s L30, L41, R63, R21, R15), *M. gordonae* (VIMS #M27), (13) *Nocardia otitidiscaviarum* (ATCC #14629), (14-16) *Enterococcus mundtii*, *Streptococcus* sp., *E. coli* (VIMS isolates), (17) negative (water) control. **3b.** RFLP (*Hin*F1/*Hpa*I) digestion patterns of lanes 2-5, respectively, showing differences in "fingerprint" pattern between species.

Water samples (200 ml) for real-time PCR analysis will be collected from 10 shallow-water sites during each 2007 ChesMMAP cruise (2/region). Sediment samples will be collected from 5 sites in the York and Rappahannock rivers during spring and summer of 2007. Such a limited sampling regime reflects the preliminary nature of this pilot study and uncertainty as to where *M. pseudoshottsii* may be distributed in the environment. Water samples will be filtered on 0.45 µm filters, and both water and sediment samples will be extracted with commercially available DNA extraction kits (DNA Stool Extraction Kit, Qiagen). Preliminary optimization of the real-time PCR assay for IS2404 has been performed by spiking known concentrations of *M. pseudoshottsii* into York River water (YRW), filtering spiked samples, and performing real-time PCR on filter extracts. A sample standard curve of known *M. pseudoshottsii* concentrations is shown in Fig. 4.

Fig. 4: Standard curve of real-time PCR-based detection of *M. pseudoshottsii* spiked in York River water (YRW). Four data points are given for each ten-fold dilution of bacteria, representing duplicate real-time PCR reactions from duplicate DNA extractions. Data points represent a dynamic range of 25-250,000 bacteria/ml of YRW.



Expected benefits

Striped bass are of huge economic and cultural importance to recreational and commercial anglers alike. This is why the current epizootic of mycobacteriosis, with as many as 70% of certain portions of the striped bass population displaying disease, is of such concern to those who have an interest in fishing and the health of the Bay in general. One of the aspects of mycobacterial disease in striped bass is the high visibility of fish with severe skin ulcers, and their reduced value to those fishers who catch them. This disease has generated a huge amount of media interest in recent years, and the public and fishery managers alike demand up-to-date, accurate information as to what is happening with this disease in striped bass of Chesapeake Bay.

The ChesMMAP survey stands as the most comprehensive existing platform for monitoring the status of the mycobacteriosis epizootic in Chesapeake Bay. The survey has baywide coverage over a large temporal scale, and does not suffer from the sampling biases inherent in pound- and gillnet collection. Use of the ChesMMAP platform for study of mycobacteriosis in striped bass thus has the potential to continue providing unprecedented understanding of the disease on a population level. This survey has provided significant insights into the epizootiology of this disease from 2003-2005, and will continue to do so in future years, pending funding. Results from 2003-2005 will be submitted for peer-reviewed publication this year, and the findings from that study will be disseminated to the public via meetings, news media, and internet resources. Continued survey of mycobacteriosis in Chesapeake Bay striped bass will have several benefits. First, accurate year-to-year information as to the status of the striped bass population with respect to mycobacterial disease will be gained. The importance of this information is illustrated by the findings from 2003-2005, which indicated the disease was in a "steady-state" within the Bay. This contradicts findings of smaller, less comprehensive studies, and provides fisheries managers and the general public with accurate information about the mycobacteriosis epizootic in striped bass. Second, although disease remained constant from 2003-2005, this does not mean the disease will remain constant in the future. Continued monitoring will provide us with a means to know whether disease becomes significantly worse or resolves at the population level. Additionally, continued monitoring will allow us to track the prevalence and severity of disease in specific components of the striped bass population over time. Coupled with natural mortality data currently being collected by other studies, this will allow us to more accurately model the disease in the striped bass population, as well as to predict what effects management practices may have. Finally, although no management practices are currently in place to control mycobacteriosis in Chesapeake Bay striped bass, further understanding of disease ecology may lead to such practices in the future. In order to gauge the effectiveness of any management strategy, it is important to have good baseline data by which to compare post-management outcomes. This proposed study will provide such data in a comprehensive, continuous manner.

Determination of which *Mycobacterium* spp. cause disease in striped bass is crucial to basic understanding of the disease in Chesapeake Bay. Culture and biochemical characterization of mycobacteria are prohibitively time- and labor-consuming, and are therefore not practical with large numbers of fish. Molecular tools such as PCR/RFLP allow rapid and accurate detection and species determination of mycobacteria from wild striped bass on a large scale. This ability is a significant step forward in our ability to understand the disease at a population level. Molecular data from this survey will also be highly valuable in directing future controlled laboratory studies. Data from the current survey, funded by VMRC, confirms the findings of previous studies that *M. shottsii* and *M. pseudoshottsii* are the mycobacterial species most commonly associated with disease in striped bass. Contrary to prior studies, however, the latter species was much more commonly detected than the former. This finding is supported by culture-derived data from fish collected during 2005. This may indicate a shift in the causative agents of mycobacteriosis in striped bass over time, or it may indicate that different *Mycobacterium* spp. affect striped bass at different ages or in different regions. We propose here to scale back the scope of molecular analysis of *Mycobacterium* spp. present in striped bass tissues, but to continue monitoring which species are associated with disease. This will provide us with further information by which to understand the disease in the striped bass population, and will further strengthen future laboratory investigations.

In addition to continuing existing datasets on the prevalence, severity, and etiologic agents of mycobacteriosis in Chesapeake Bay striped bass, this study will provide important data on the ecology of mycobacteriosis in the Bay. Additional finfish species, including flounder, weakfish, spot, croaker, white perch, and menhaden, will be examined for the presence of *Mycobacterium* spp. that also infect striped bass. This will give us information as to potential routes of exposure for striped bass, as well as identifying additional finfish species that may be at risk for

disease. To date, the distribution of mycobacteria such as *M. shottsii* and *M. pseudoshottsii* in the environment is completely unknown. Toward a holistic understanding of the disease process in Chesapeake Bay, this is a crucial piece of information. The proposed study will begin to examine this question in a pilot survey. Assuming *M. pseudoshottsii* is present in the environment (water or sediments), the techniques applied here will be of paramount importance in determining the natural and manmade factors that influence its distribution. This information has the potential to be of great relevance to future management efforts targeted at reducing exposure of striped bass to disease-causing agents.

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Results of prior VMRC/RFAB funding

Research projects to study the prevalence and severity of mycobacteriosis in Chesapeake Bay striped bass, as well as to develop molecular tools for detection and species determination of mycobacteria in striped bass tissues, have been supported by the VMRC/RFAB with grants in 2004 and 2005. A final report will be delivered to the board for the 2005 grant, and a manuscript is currently in preparation detailing findings from the ChesMMAP survey from 2003-2005. We present here some representative data from our studies to date.

A major goal for the project funded by VMRC in 2005 was to develop and implement molecular tools to detect and determine the species of mycobacteria present in striped bass tissues. These studies are ongoing, and a final report will be delivered to the RFAB in November of this year. Preliminary studies were performed in spring and fall of 2005 in collaboration with the United States Geologic Survey. Striped bass were collected via the ChesMMAP platform and analyzed for mycobacterial infection and disease with histological, bacteriological, and molecular methods. Some preliminary data is presented below in Table 1. Among PCR-positive samples, the majority demonstrated RFLP patterns consistent with infection by *M. pseudoshottsii*. This contrasts with data presented in previous culture-based surveys, in which *M. shottsii* was the dominant isolate (Rhodes et al. 2004), and indicates that *M. pseudoshottsii* must be considered as an important potential disease agent in striped bass.

Table 1: PCR/RFLP analyses of striped bass spleens collected during joint USGS/ChesMMAP survey during spring and fall, 2005. Column headings are as follows: MPS=*M. pseudoshottsii*, MS=*M. shottsii*, MM=*M. marinum*, MPS/MS=dual *M. pseudoshottsii/M. shottsii*, ?=positive at genus level, but not identified, MTX="*M. triplex*-like" group. "Other" indicates an apparent MPS/MS/MM polyinfection observed in one fish from the Fall 2005 sample.

	MPS	MS	MM	MPS/ MS	?	MTX	Other	Total
Spring 2005	19	1	2	1	2	2	0	27
Fall 2005	49	2	0	10	2	0	1	64

Striped bass have a highly complex life history, with distinct populations of nonmigratory younger fish and older fish whose migrational range covers the entire Chesapeake Bay. To gain a more complete understanding of mycobacterial disease in Chesapeake Bay, it is necessary to examine disease prevalence in both male and female fish in as many age groups as possible and over broad spatial and temporal scales. This necessity is demonstrated by data collected by the ChesMMAP survey from 2003-2005. As shown in Fig. 5, disease prevalence is dependent on both age and sex of striped bass. One of the most striking findings from this data is that while disease prevalence is similar in male and female fish until approximately age 6, a major contrast is observed between sexes in fish age 7 and older. There are several possible explanations for this finding. The possibility that male fish are inherently more susceptible to disease is unlikely, based on the similarity in prevalence observed between sexes in age ≤6 fish. Disease (and therefore infection) is observed in age 1 fish, which can be presumed not to have left Chesapeake Bay for coastal migration (Mansueti 1961). Therefore, both exposure and factors necessary for disease expression are found within Bay waters. Striped bass are sexually dimorphic both with respect to size and migratory behavior, with female fish typically beginning to leave the Bay at age 2+, while male fish do not migrate to an appreciable extent until at least age 4 (Kohlenstein 1981, Dorazio et al. 1994). Thus, male fish as a whole spend more time in Bay waters, and are thus presumably exposed to risk factors for disease for a longer period of time than females. The fact that apparent prevalence for both male and female fish increased until age 6, however, coupled with the lack of knowledge about what disease risk factors may exist outside the Bay, makes this argument somewhat suspect, and much better information regarding the residence time and distribution of male and female striped bass in Chesapeake Bay is necessary to further evaluate it. Further, this argument would enjoy greater support if prevalence

among female fish continued to increase with age, but at a lower rate than males. The fact that apparent prevalence decreased in age 7+ females relative to younger females suggests that additional mechanisms are at work. The observed decrease may be due to mortality of diseased females, regression/healing of disease (e.g. through lack of exposure to risk factors), or differential migration of diseased fish. It is generally assumed that mycobacteriosis in fishes, while chronic, is ultimately fatal, and regression of disease in laboratory exposure studies has not, to our knowledge, been observed. The ability of mycobacteria of human and veterinary importance to enter latency, however, is well known (Flynn & Chan 2001), and may partially explain the sex-specific apparent prevalence observed in this study.

Table 2 presents a logistic regression analysis of the 2003-2005 prevalence and severity data. Logistic regression is a specialized statistical method by which the influence of many variables (in this case age, cruise, and region) may be simultaneously analyzed for their effect on an outcome (in this case presence of disease). To illustrate why this type of analysis is useful in this case, consider a case in which disease prevalence is found to be higher in one region than another. This increased prevalence may be due to an actual regional effect, or it may be due to the fact that there were more older fish (which have higher disease prevalence) in that region. Logistic regression takes these issues into account, and assigns an "odds ratio" to each variable that is corrected for the other variables. If the odds ratio is significantly above 1 (as indicated by bold type in Table 2), this indicates that a variable is a risk factor for disease. If the odds ratio is significantly below 1 (as indicated by italics in Table 2), this indicates that a variable is negatively related to risk for disease. From the 2003-2005 data, the effect of age on disease prevalence is very clear, with risk increasing with age for both male and female fish until age 6. In older (age 7+) fish, risk of disease for males is greatly elevated, whereas there is a reduction in risk for female fish almost down to the level of young (age 1-2) fish. This is the same pattern that is seen in simple apparent prevalence data (Fig. 5).

Information on seasonal (cruise) and geographical (region) effects on prevalence of mycobacteriosis are also presented in Table 2. Relative to region 5, a significant reduction in risk of disease was seen in regions 1 (OR 0.314, 0.623-0.158) for male fish, and a marginally significant increase in risk was seen for female fish in region 3 (OR 2.326, 5.378-1.006). These data indicate that male fish in the northern regions of the Bay are at less risk for disease than in the rest of the Bay. Interpretation of these findings, however, is complicated by the fact that striped bass are highly migratory. The reduction in risk for northern Bay males may be related to lower exposure to disease risk factors, lower rates of migration, or a combination of the two.

Relative to cruise 5, significant reduction in disease risk was observed in region 1 for both male and female fish (OR_{male} 0.346, 0.52-0.231; OR_{female} 0.447, 0.756-0.265), and in region 2 for male fish (OR 0.404, 0.645-0.254). These data indicate that, when corrected for age, striped bass have a lower risk for disease earlier in the year than later. This likely indicates increasing disease expression as the year progresses, but also implies that some mortality of diseased fish occurs over winter months. Alternately, healing and regression of disease may occur over the winter, resulting in lower rates of apparent prevalence in the spring. Ongoing disease-related mortality studies will likely shed more light on these possibilities.

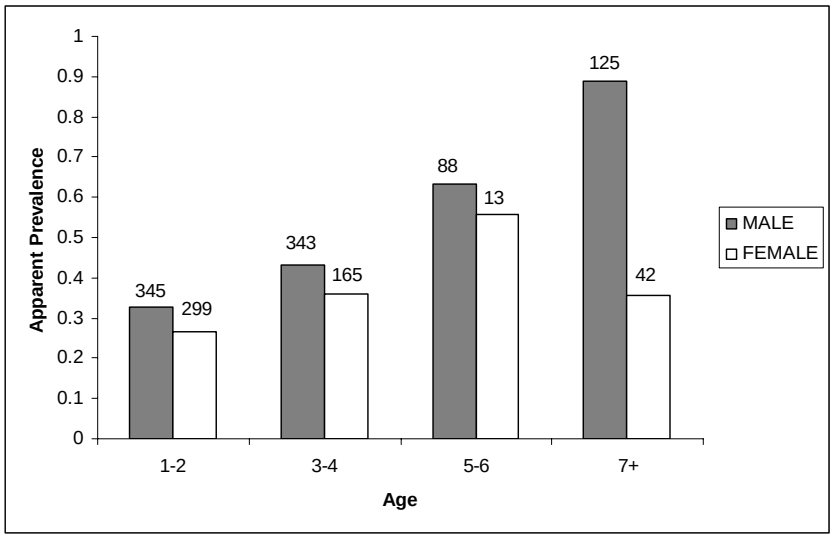

Fig. 5: Apparent prevalence of mycobacteriosis for male (gray bars) and female (clear bars) striped bass; 2003-2005 pooled data.

Table 2: Relationship between age category, cruise, region and apparent prevalence of splenic mycobacteriosis in striped bass, pooled 2003-2005 data. Odds ratios (OR) significantly >1 are in bold type, OR significantly <1 are in italics. Geographical regions are shown below.

Sex		Parameter	OR	95%CI	*p*
Male	Age	7+	**22.088**	**42.327-11.526**	**<0.001**
		5-6	**6.485**	**11.316-3.716**	**<0.001**
		3-4	**4.347**	**6.23-3.033**	**<0.001**
		1-2	Reference		
	Cruise	1 (March)	*0.346*	*0.52-0.231*	*<0.001*
		2 (May)	*0.404*	*0.645-0.254*	*<0.001*
		3 (July)	0.612	1.207-0.31	0.156
		4 (September)	1.059	2.151-0.521	0.875
		5 (November)	Reference		
	Region	1	*0.314*	*0.623-.158*	*0.001*
		2	0.692	1.288-0.371	0.245
		3	0.978	1.83-0.522	0.944
		4	0.973	1.894-0.5	0.936
		5	Reference		
Female	Age	7+	**2.304**	**4.723-1.124**	**0.023**
		5-6	**42.913**	**349.195-5.274**	**<0.001**
		3-4	**4.687**	**7.52-2.922**	**<0.001**
		1-2	Reference		
	Cruise	1	*0.447*	*0.756-0.265*	*0.003*
		2	0.714	1.539-0.331	0.39
		3	0.922	2.258-0.376	0.858
		4	1.067	2.457-0.464	0.878
		5	Reference		
	Region	1	1.093	2.75-0.434	0.85
		2	1.414	3.245-0.616	0.414
		3	**2.326**	**5.378-1.006**	**0.048**
		4	1.775	4.241-0.743	0.196
		5	Reference		

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